Discovering biomarkers and druggable targets in uveal melanoma

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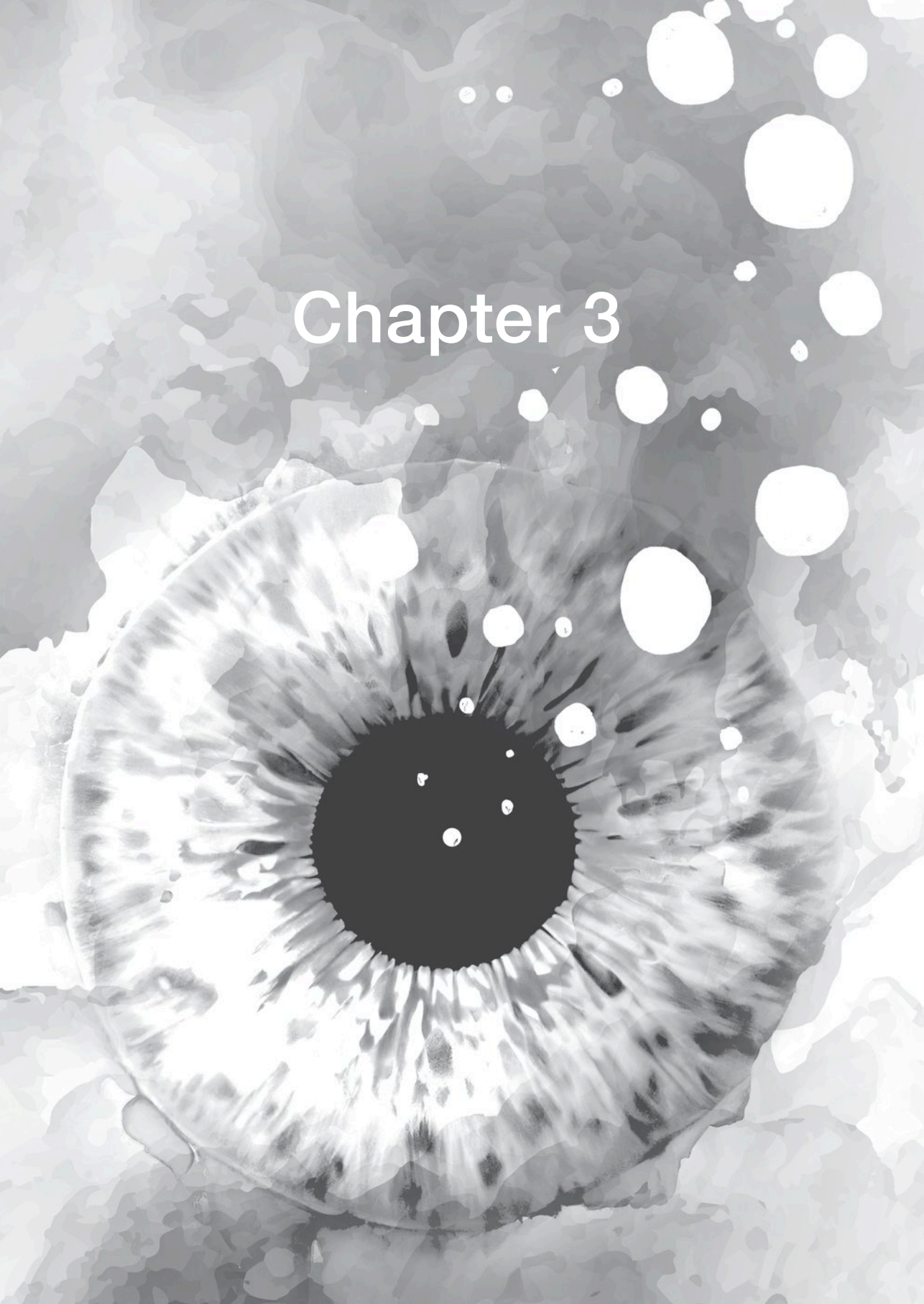
Chapter 3

Immune Checkpoint Inhibitors in Uveal and Conjunctival Melanoma.

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Immune Checkpoint Inhibitors

Many therapies are being developed that use the patient's own immune response to treat malignancies. One of the new types of immunotherapy is the application of monoclonal antibodies that stimulate immune responses. The 2018 Nobel Prize in Physiology or Medicine was awarded to James P. Allison and Tasuku Honjo "For their discovery of cancer therapy by inhibition of negative immune regulation". Dr Allison determined the function of a protein expressed on T cells called CTLA4 and Dr Honjo on PD1, and both researchers discovered that contact of a T cell with the ligand of these proteins would inhibit activation of tis T cell. CTLA4 and PD1 may thus be considered to function as "brakes" of immune responsiveness; these molecules are known as immune checkpoints. However, they can be modulated: blocking of the immune checkpoints stops their function. When the immune checkpoints are blocked, they no longer inhibit the immune response, which allows T cells to attack their target, for example tumor cells. Immune checkpoints can be blocked with monoclonal antibodies; well-known monoclonal antibodies are ipilimumab (directed against the CTLA4 on T cells), nivolumab and pembrolizumab (both directed against the PD1 receptor on T cells) and atezolizumab (directed against PD-L1, the ligand of PD1).

Activating the Immune Response

In order to get an effective anti-tumor T-cell response, T cells need to have their T-cell receptor interact with their antigen, presented on a major histocompatibility molecule of an antigen-presenting cell. In addition, co-stimulatory signals have to stimulate the T cell, as for instance happens when CD28 on the T cell interacts with CD80 or CD86 on an antigen-presenting cell. However, at this point, inhibitory signals may counteract this stimulation: when CTLA4 binds to its ligands (also CD80 or CD86) or PD1 binds to PD-L1 (also known as B7-H1 or CD274), a negative signal is generated, which inhibits the T cell. Either the negative or positive signal wins (mechanism reviewed by Sharpe and Pauken 2018) [1].

Clinical Trials in Cutaneous Melanoma

Activation of anti-T-cell responses is being used to treat many malignancies, with sometimes excellent success. An important early phase III study in 676 patients with metastasized cutaneous melanoma (stage III or IV) compared three treatment: ipilimumab alone (3 mg/kg), a gp100 peptide vaccination, and a combination of the two. Overall survival in the three groups was 10.1, 6.4 and 10.0 months, respectively [2], showing that the treatment of ipilimumab was better than the then optimal available therapy.

Checkpoint inhibitors may also be used as adjuvant treatment in cutaneous melanoma. In order to prevent recurrences in high risk patients, Eggermont et al. [3] treated patients who had undergone a complete resection of a stage III cutaneous melanoma; the patients were given a dose of 10 mg/kg ipilimumab (475 patients) or placebo (476 cases) every 3 weeks for four doses, followed by one dose every 3 months for up to 3 years or until disease recurred or toxicity was too high. At a median follow-up of 5.3 years, the 5-year rate of recurrence-free survival was 40.8% in the ipilimumab group versus 30.3% in the placebo group. Five patients in the ipilimumab group died from immune-related adverse effects.

Checkpoint Inhibition in Uveal and Conjunctival Melanoma (CM)

When looking at the overall survival of patients (n =1102) enucleated for a uveal melanoma (UM) at the LUMC between 1972 and 2017 (Figure 1), it is obvious that therapies that attack the development of metastases are urgently needed. However, as yet, success of checkpoint inhibitors in UM is limited, while the first good responses have been identified in patients with metastases in CM. We look at expression of some of these molecules and describe their expression, as well as some current trials for UM and CM.

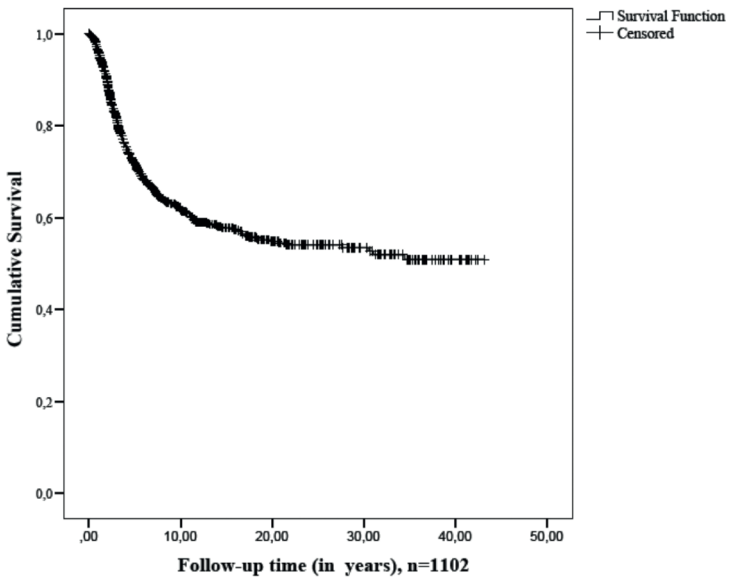

Figure 1. Overall survival of 1102 patients treated with an enucleation for a uveal melanoma in the LUMC, between 1972 and 2017.

In Vitro Targeting of Checkpint Inhibitors in CM and UM

Ocular melanoma can develop as conjunctival melanoma (CM) on the ocular surface, or inside the eye as a UM, being derived from uveal tissues: the iris, ciliary body and choroid. Those that develop in the ciliary body or choroid give rise to metastases, which will lead to the death of around 50% of UM patients. In recent years, the discovery of immune checkpoint inhibitors has led to many new treatments. UM patients have regrettably not benefitted from the new immune checkpoint inhibitors, while they seem to help patients with CM metastases.

Tumors have many ways to evade immune responses, such as by secreting cytokines such as TGFbeta, or expressing T-cell inhibitors. Well-known T cell inhibitors are CTLA4 and PD-L1. The receptor for PD-L1 is PD1, which is expressed on thymocytes, mature T and B cells, and myeloid cells such as macrophages. When the PD1 receptor on T cells binds to its ligand, PD-L1, the activation and subsequent proliferation and production of cytokines by the T cell are blocked. Tumors cells may express PD-L1 to prevent their killing by the T cell.

PD-L1 Expression in UM

Yang et al. [4] performed an extensive study of the function of PD-L1 and PD1 in UM in vitro. Expression was determined on six cell lines derived from primary UM and five cell lines from UM metastases. The effect on IL-2 production by T cells was measured using the interaction between Jurkat T cells and in vitro-grown melanoma cells.

Fluorescence-activated cell soring analysis demonstrated expression of PD-L1 on 3/6 primary UM cell lines and 1/5 metastasis-derived cell lines, while expression increased on all cell lines by adding interferon gamma (500 U/ml). While there was a low native expression of PD-L1, a significant upregulation of expression was seen after addition of interferon gamma. Co-culture of stimulated Jurkat cells with cell line OCM1 inhibited the production of IL-2 by the Jurkat cells, while addition of an anti-PD-L1 monoclonal antibody led to restoration of the IL-2 production. The experiments clearly show the inhibitory function of the PD-L1 - PD1 interaction in melanoma cells, by demonstrating the inhibitory effect on T cell activity, and the effective application of anti-PD-L1 antibody in restoring T cell recognition and activity. A very interesting observation was made with regard to the interaction of UM cell line Mel290 and PD-L1: while cultured MEL290 cells did express high levels of PD-L1, they were not able to suppress the production of IL-2 by the Jurkat cells. However, treatment with interferon gamma restored the capacity to suppress the production of IL-2. The same phenomenon was observed for corneal endothelial cells [5]. It seems that an extra interferon gamma-induced signal is needed to make the PD-L1

signal effective. Since the more malignant UM, that give rise to metastases, express an interferon gamma-associated inflammatory phenotype, it may well be that especially these more malignant UM are more effective in preventing T-cell mediated lysis. We published that the inflammatory phenotype is associated with monosomy of chromosome 3, a high risk factor for the development of metastases [6-8]. This may be a difference with other tumors, where the presence of an infiltrate is a good prognostic indicator.

Yang analyzed expression on five UM tissue sections but did not see any expression of PD-L1 on the primary tumor. This absence of staining may be antibody dependent (clone MIH1, eBioscience), as another study did demonstrate expression: Ma et al. [9] studied the differential effects of triggering different co-stimulatory molecules on UM cells. While some cell surface molecules function as brakes, others function as stimulators. The role of B7-H1 (old name of PD-L1) was studied by using a blocking antibody, and the effect of CD40 (a stimulator) was assessed using recombinant CD40 ligand. While three different cell lines upregulated B7-H1 after incubation with interferon-gamma, one of the cell lines (92.1) did not express CD40 and its expression was not increased after addition of interferon gamma to the culture system. The other two cell lines secreted increased levels of IL-8, MCP-1, IP-10 and RANTES after stimulation with rhCD40L. These data again indicate that different cell lines differ in their response to gamma interferon, making them either more resistant or more sensitive to T cells. It may well be that, similar to the cell lines, different tumors might also show a differential response to external stimuli, based on their receptor expression, leading to a differential sensitivity to immune regulators such as checkpoint inhibitors or stimulators.

Expression of PD-L1 and PD1 in Eyes

Zoroquiain et al. [10] focused on the expression of PD-L1 and PD1 on UM tissues as a potential target for therapy. When 5% positivity was used as a threshold, 46% of cases showed PD-L1 expression (clone E1L3N). Of the 67 evaluable patients, 25 (37%) developed metastases, with a median follow-up of 24 months. In the control group, the median follow-up was 58 months. Significantly shorter metastasis-free survival was associated with a mixed cell type, the presence of tumor-associated macrophages, tumor-infiltrating lymphocytes, and ciliary body involvement. PD-L1 expression on tumor cells was negatively associated with the number of tumor-infiltrating lymphocytes. A total of 43% of the tumors had constitutive PD-L1 expression. This negative association between expression of PD-L1 and infiltrating T cells is quite surprising, as the in vitro experiments has shown that T cells may secrete interferon gamma which is known to upregulate PD-L1). Patients with tumors that showed

expression of PD-L1 on either tumor cells or infiltrating immune cells (36 cases) had a better metastasis-free survival than those that did not (31 cases) (P = 0.038).

This study was performed on enucleated eyes, but immunohistochemical analysis can also be performed on biopsies; however, it would be more practical if one could analyze ocular fluids, as they are more easily obtained than biopsies.

PD-L1 Expression in the Aqueous Humor

We therefore determined whether one can use anterior chamber fluid to determine the presence of soluble PD-L1 in anterior chamber fluid in eyes that had been enucleated between 1999 and 2017 at the LUMC. Directly following enucleation, an anterior chamber puncture had been performed using a 23-g needle, to collect a sample of aqueous humor. The measurement of PD-L1 in the aqueous humor was performed as part of an assay with other immunological and tumor biomarkers (Olink® Immuno-Oncology panel, from Olink Proteomics AB, Uppsala, Sweden). The Proximity Extension Assay (PEA) technology used for the selected Olink assay has been described [11], and enables 92 proteins to be analyzed simultaneously, by using the amount of 1 µL per sample: pairs of oligonucleotide-labelled antibody probes bind to their targeted protein, and if the two probes are brought in close proximity the oligonucleotides will hybridize in a pair-wise manner. The addition of a DNA polymerase leads to a proximity-dependent DNA polymerization event, generating a unique PCR target sequence. The resulting DNA sequence is subsequently detected and quantified using a microfluidic real-time PCR instrument (Biomark HD, Fluidigm). Data is then quality controlled and normalized using an internal extension control and an inter-plate control, to adjust for intra- and inter-run variation. The read-out is presented in Normalized Protein eXpression (NPX) values, which is an arbitrary unit on a log2-scale where a high value corresponds to a higher protein expression.

The data on PD-L1 showed a wide range (Figure 2), and we compared expression levels with clinical, histopathological and genetic markers (Table 1). Analysis of the group showed that PD-L1 levels were significantly higher in relation with old age (P=0.006), a high prominence (P<0.0001) and diameter (P=0.01) and involvement of the ciliary body (P<0.0001). Conformingly, the PD-L1 expression levels increased with every stage of the AJCC staging (P<0.0001). PD-L1 expression was significantly higher in tumors with an epithelioid or mixed cell type (P=0.024). With regard to chromosome status, PD-L1 expression tended to be higher in the aqueous of tumors with monosomy of chromosome 3 (P=0.072) and tumors with gain of 8q (P=0.005).When looking at survival, patients with a high PD-L1 expression had a significantly worse survival (Log

Rank, P=0.001, Figure 3). This contradicts the findings as reported by Zoroquiain et al. (2018) but fits with prior findings that the presence of an inflammatory phenotype is associated with a bad prognosis.

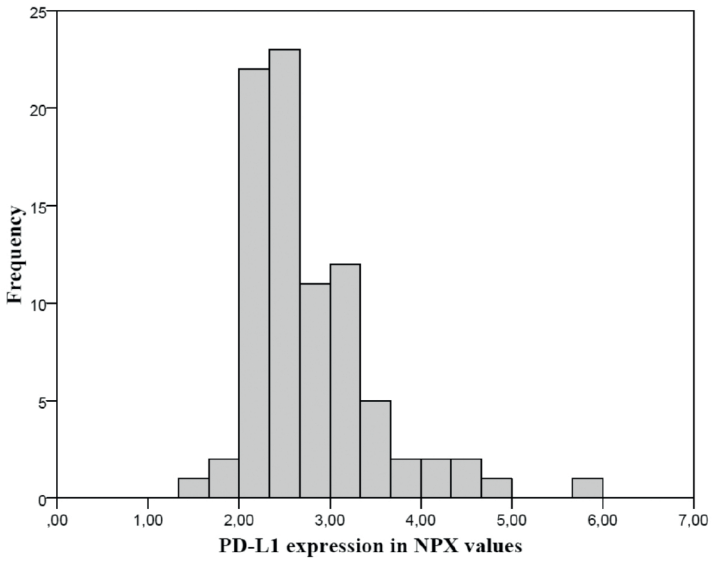

Figure 2. Distribution of PD-L1 expression in the aqueous humor of 84 patients included in the study. NPX indicates Normalized Protein eXpression.

Clinical studies

Several clinical studies included UM patients with metastases. In a 2018 review of the literature, Jindal reviewed series that used ipilimumab (anti-CTLA4), nivolumab (anti-PD1), atezolizumab (anti-PD-L1), tremelimumab (anti-CTLA4) and pembrolizumab (anti-PD1), sometimes in combination [12]. Series varied between 5 and 96 cases. The median overall survival was between 3 and 12.8 months, with the response rates in the series with more than 25 cases being between 0 and 7.7%.

One specific case has drawn the attention of immunologists: the patient showed an exceptional high sensitivity to PD1-inhibitor pembrolizumab, and was subsequently identified to have a hypermutated phenotype with many somatic single nucleotide variants, due to especially CpG>TpG mutations. Subsequent gene analysis revealed a germline mutation in *MBD4* [13], with loss of the other gene in the tumor due to monosomy 3. A similar case was found in the TCGA cohort [14]. The function of *MBD4* is to protect the DNA from methylation damage.

Table 1. Association between PD-L1 levels in aqueous humor and clinical, histopathological and chromosome characteristics of the study population.

Clinical and Histopathologic characteristics	*n*	Mean (±SD)	Mean PD-L1 expression in NPX (±SD)	*P* Value
Gender (n = 84)				0.413[c]
Male	46		2.79 (±0.70)	
Female	38		2.74 (±0.75)	
Age at enucleation in years	84	59.7 (±15)		0.006[b]
Tumor prominence, mean in mm	83	7.6 (±2.9)		<0.0001[b]
Tumor diameter in mm	83	12.9 (±2.9)		0.010[b]
Ciliary body involvement (n = 84)				<0.0001[c]
No	55		2.53 (±0.43)	
Yes	29		3.22 (±0.92)	
Eye colour (n = 58)				0.386[c]
Light (blue, grey, green, hazel)	45		2.78 (±0.69)	
Dark (brown)	13		3.14 (±0.93)	
Mitotic count * mean (±SD)	77	5.16 ±4.7		0.289[b]
Histopathologic cell type (n = 84)				0.024[c]
Spindle cell	28		2.52 (±0.41)	
Epithelioid or mixed cell type	56		2.90 (±0.80)	
Necrosis (n = 83)				0.003[c]
No	63		2.65 (±0.66)	
Yes	20		3.15 (±0.77)	
Pigmentation of the tumor (n = 80)				0.287[c]
None to limited	44		2.70 (±0.75)	
Moderate to intense	36		2.83 (±0.69)	
Bruch's membrane broken (n = 79)				0.648[a]
Unclear	12		2.78 (±0.79)	
Intact	16		2.63 (±0.59)	
Broken	51		2.85 (±0.75)	
AJCC Stage (n = 84)				<0.0001[a]
I	7		2.33 (±0.37)	
IIA	26		2.40 (±0.29)	
IIB	22		2.74 (±0.39)	
IIIA-IIIC	26		3.22 (±0.91)	
IV	2		4.14 (±0.32)	
Vital status (n = 84)				0.017[a]
Death due to UM metastases	37		2.91 (±0.67)	
Death due to other causes	13		2.44 (±0.35)	
Alive at last follow-up date	34		2.74 (±0.83)	
Monosomy 3 status (n = 81)				0.072[c]
No	31		2.56 (±0.43)	
Yes	50		2.89 (±0.81)	
Chromosome 8q status (n = 74)				0.005[c]
No gain of 8q	22		2.42 (±0.37)	
Gain of 8q	52		2.87 (±0.79)	
BAP1 staining (n = 71)				0.035[c]
Normal	31		2.62 (±0.56)	
Deficient	40		2.92 (±0.67)	

[a] Kruskal-Wallis Test
[b] Spearman's rho correlation
[c] Mann-Whitney U test
* Number of mitoses/mm2 per 8 high power fields (hpf)

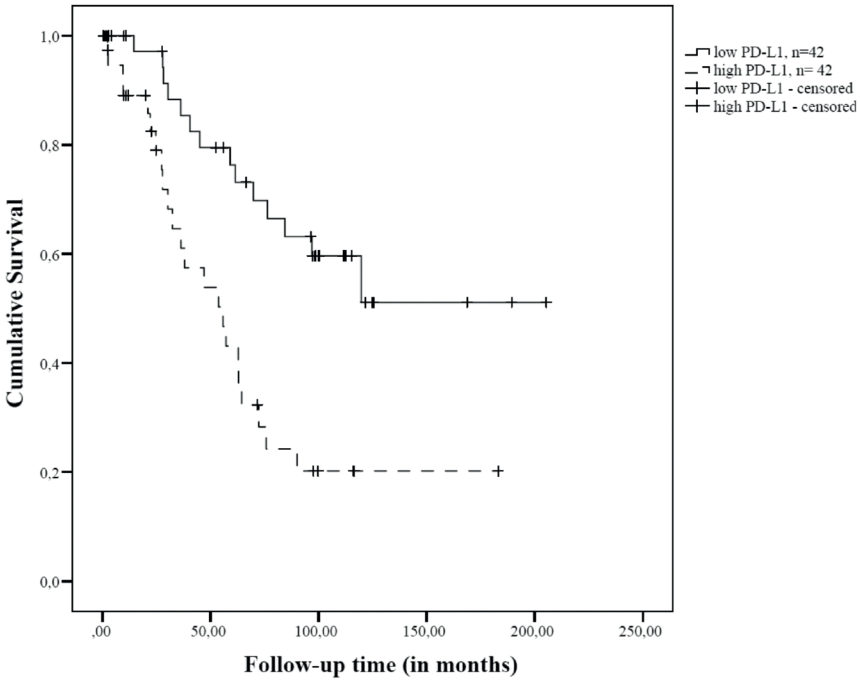

Figure 3. The cumulative survival of uveal melanoma patients with a low (n =42) or high (n =42). PD-L1 expression in their aqueous humor, analyzed at the time of enucleation, separated according to the mean (P =0.001, Log Rank test).

Conjunctival melanoma

CM is a rare disease, which is, however, associated with high local (61% after 5 years) and systemic recurrence rates (14%, [15]). Genetically, these tumors resemble cutaneous melanoma, as they carry *BRAF*, *NRAS* and *Kit* mutations [16-18], while uveal melanoma are known to carry *GNAQ* or *GNA11* oncogenic mutations and secondary mutations in *BAP1*, *SF3B1,* or *EIF1AX* [19].

Cao et al. [20] analyzed 27 cases of CM with immunofluorescence staining for PD-L1 and PD-1, and observed that PD-L1 expression could occur on stromal cells or tumor cells. PD-L1 was expressed on at least 5% of the cells of 5/27 tumors and on stromal cells in 16 tumors (59%). Double staining demonstrated that PD-L1 was often expressed on M2 macrophages. PD1 expression on T cells was noticed on 17 (63%) tumors. PD-L1 expression on the tumor cells was associated with the development of metastases and death.

Three CM cell lines could be analyzed and two out of three expressed PD-L1, which was further upregulated following exposure to interferon gamma. Saqiv et al. [21] recently described five patients with CM metastases who had been treated with checkpoint inhibitors, with promising results.

Side Effects

While the treatment of malignancies with immune checkpoint inhibitors has been met with great success, the function of these antibodies has an inherent downside, as their use may lead to severe clinical side effects. While blocking of the immune checkpoint inhibitors allows anti-tumor T cells to respond against tumor cells, this treatment at the same time releases T cell responses against normal tissues. A wide range of auto-immune diseases are associated with the use of anti-CTLA4, anti-PD1 and anti-PD-L1 antibodies. In the study described by Hodi et al. [2], ocular side effects were already observed. In the series of cases of cutaneous melanoma that received ipilimumab as adjuvant therapy, five patients died from drug-related complications [3]. Among the ocular side effects of checkpoint inhibitors are dry eyes, conjunctivitis, episcleritis, keratitis, anterior and posterior uveitis and orbital inflammation [22-24].

That PD1 and PD-L1 are relevant to ocular immune privilege was demonstrated by several murine studies. PD1 is constitutively expressed in the eye [25]. In a mouse model of corneal transplantation, cornea transplants underwent rejection when PD1 or PD-L1 were blocked [26]. Under normal conditions, PD-L1 is not expressed on retinal pigment epithelial cells, but it is when exposed to interferon-gamma. PD-L1 expressing retinal pigment epithelium cells are able to suppress T cell activation [27]. It is likely that the expression of these checkpoint inhibitors plays a role in ACAID (Anterior Chamber-Associated Immune Deviation), and that the infusion of antibodies that block the checkpoint inhibitors are able to release existing T-cell responses against intraocular tissues, in spite of other immunosuppressive mechanisms in the eye.

Conclusions

While immune checkpoint inhibitors are being used successfully in many malignancies, first results show that they may be effective in cases of CM. The majority of UM patients do not respond favorably, although the different targets are expressed in primary UM and can then even be found in the eye.

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